Determination of the Autoxidation Products from Free or Total Cholesterol: A New Multistep Enrichment Methodology Including the Enzymatic Release of Esterified Cholesterol

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A new method for the determination of the autoxidation products from free cholesterol, viz., the isomeric 5,6-epoxycholestanols, the C-7 oxidized cholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, and cholestanetriol, has been developed. The method is also useful in combination with an enzymatic method to release esterified cholesterol to facilitate total cholesterol oxide determination. For analysis of total cholesterol oxides, the lipids were dispersed in phosphate buffer by using bovine serum albumin as a detergent and incubated with triacylglycerol lipase under nitrogen atmosphere. The phospholipids and the released fatty acids were removed by column chromatography. The remaining lipids were dispersed again and incubated with cholesterol ester hydrolase under nitrogen atmosphere. Cholesterol oxides were isolated in three enrichment steps. The isolated cholesterol oxides were separated as trimethylsilyl ether derivatives on a capillary methyl silicone column.

INTRODUCTION

Cholesterol, an unsaturated compound, undergoes autoxidation in the presence of molecular oxygen through a free-radical reaction, predominantly at position C-7. An array of primary and secondary oxidation products is thus formed. Some 70–80 of the oxidation products have been partially or fully identified (Smith, 1981, 1987). A number of the oxidation products have adverse biological effects in vivo and in vitro such as cytotoxicity, angiotoxicity, carcinogenicity, and mutagenicity. These biological properties are discussed in three comprehensive reviews (Smith, 1981; Addis et al., 1983; Peng and Taylor, 1984).

A number of methods have been developed for the quantitative determination of the cholesterol oxides in various food products exposed to heat and air during manufacturing and/or long-term storage, but no standard method as yet exists which combines the essential qualities of accuracy, precision, and selectivity with that of speed. Primary attention has been focused on the isomeric 5,6-epoxycholestanols (Tsai and Hudson, 1984; Sugino et al., 1986) or the epimeric 7-hydroxycholesterols and 7-ketocholesterol (Herian and Lee, 1985; Park and Addis, 1985). In contrast, few studies have analyzed 20α-hydroxycholesterol, 25-hydroxycholesterol, and cholestanetriol in addition to the above-mentioned cholesterol oxides. Missler et al. (1985) developed a method for the quantitative determination of all the biologically important cholesterol oxides in egg mix powder. This method involved enrichment of the cholesterol oxides by chromatography on a silica gel 60 column, followed by high-performance liquid chromatography using a silicic acid column to remove the cholesterol and finally capillary gas chromatography analysis of the cholesterol oxides as trimethylsilyl ether (TMS ether) derivatives.

In the author's laboratory, the removal of the triacylglycerols, other nonpolar lipids, and almost all of the cholesterol was achieved in a single step using a Lipidex 5000 column. The phospholipids and free fatty acids were retained on an anion exchanger derivative of Lipidex 5000, triethylaminohydroxypropyl-Lipidex (Nourooz-Zadeh and Appelqvist, 1987). However, this method is too cumbersome and tedious to be employed routinely.

Saponification has commonly been used as an enrichment step in the quantitative determination of the autoxidation products from total cholesterol. Hot saponification followed by column chromatography on Al_2O_3 and subsequently a silica gel 60 column were used by Fischer et al. (1985). The cholesterol oxides were then separated as TMS ether derivatives on a capillary gas chromatography column. However, hot alkaline treatment of cholesterol and its autoxidation product is a significant source of error in the quantitation of cholesterol oxides. Tsai et al. (1980) reported a loss of 75% of the 5α,6α-epoxycholestanol after saponification according to the official American Oil Chemist Society (AOSC) method Tk 1a-64T. Maerker and Unruh (1986) reported no losses of the isomeric 5,6-epoxycholestanols using the Association of Official Analytical Chemists (AOAC) saponification method 28:092 or the dry column saponification according to the method of Maxwell and Schwartz (1979) but did report a substantial loss of 7-ketocholesterol. In contrast, Park and Addis (1986) reported complete recoveries of the 5α,6α-epoxycholestanol and 7-ketocholesterol with or without tallow after incubation with 1 M KOH for 18 h at room temperature.

No reliable data on esterified cholesterol oxides in foods have yet been reported. The aims of the present study were (a) to develop a more rapid method for the quantitative determination of a wide range of autoxidation products from free cholesterol and (b) to develop a mild enzymatic method for the release of esterified cholesterol oxides followed by a determination of the sum of free and released cholesterol oxides. The present study was part of a Ph.D. thesis (Nourooz-Zadeh, 1988).

MATERIALS AND METHODS

Food Samples. Fresh eggs were purchased from a local supermarket. Spray-dried egg yolk powder was obtained from AB Svenska Äggprodukter, Helsingborg, Sweden.

Reagents. Cholest-5-en-3β-ol (cholesterol), cholest-5-en-3β-ol oleate (cholesteryl oleate), 5,6α-epoxy-5α-cholestan-3β-ol

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(5α,6α-epoxycholestanol), 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), 3β-hydroxycholest-5-en-22-one (22-ketocholesterol), cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), cholest-5-ene-3β,20α-diol (20α-hydroxycholesterol), cholest-5-ene-3β,25-diol (25-hydroxycholesterol), and 5α-cholestane-3β,5,6β-triol (cholestanetriol) were obtained from Sigma Chemical Co., St. Louis, MO, and Steraloids Inc., Wilton, NH. 5,6β-Epoxy-5β-cholestan-3β-ol (5β,6β-epoxycholestanol), ^{3}H-labeled cholesterol, and ^{14}C-labeled 5,6-epoxycholestanol were provided by professor Peter Eneroth, Karolinska Institute, Stockholm, Sweden, while ^{3}H-labeled cholestanetriol was a gift from Professor Ingemar Björkhem, Department of Clinical Chemistry, Huddinge Hospital, Stockholm, Sweden. [1-^{14}C]cholesteryl oleate was obtained from Amersham, Buckinghamshire, U.K. Solvents were purchased from E. Merck, Darmstadt, FGR, with the exception of 1,2-dichloroethane, which was obtained from Fischer Scientific, Fair Lawn, NJ. Tri-Sil was purchased from Pierce Chemical, Rockford, IL. Sep-Pak C_{18} cartridges were obtained from Waters Associates, Milford, MA, and the 500-mg NH_2 cartridges (aminopropyl sorbent) from Analytichem International, Harbor City, CA. Tripamitolein (TG 16:1), triacylglycerol lipase type XI from *Rhizopus* (activity approximately 400.000 units/mg of protein) and bovine serum albumin (fraction V, powder, 98–99% albumin) were purchased from Sigma, while the triheptadecanoin (TG 17:0) was obtained from NU-Chek Prep Inc., Elysian, MN. The cholesterol esterase (from *Pseudomonas fluorescence*, activity 110%, protein 100%) was obtained from Calbiochem, San Diego, CA. The phase-combining system (PCS) for liquid scintillation was obtained from Amersham Corp., Arlington Heights, IL.

Lipid Extraction. Six egg yolks, of 5 g of spray-dried egg yolk powder, were transferred into a separation funnel, 90 mL of hexane/2-propanol (3/2 v/v) was added, and the funnel was shaken vigorously for 3 min. The suspension was centrifuged at 4000 rpm for 5 min. The supernatant was then transferred into a new separation funnel, and 60 mL of aqueous 0.47 M Na_2SO_4 was added. The mixture was agitated and subsequently allowed to settle. The upper layer, mainly hexane, was transferred into an evaporation flask and, finally, the lipids were dried in a rotary evaporator at 35 °C.

Enrichment of the Autoxidation Products from Free Cholesterol. One hundred milligrams of egg yolk lipids containing approximately 0.8 μg of 3β-hydroxycholest-5-en-22-one as an internal standard was dissolved in 1 mL of hexane/1,2-dichloroethane (1/1 v/v) and then applied on a NH_2 cartridge. The column was washed with 17 mL of hexane to remove hydrocarbons, cholesterol esters, and the majority of the triacylglycerols. The remaining triacylglycerols, cholesterol and cholesterol oxides, were then eluted by washing the column with 25 mL of hexane/1,2-dichloroethane/2-propanol (55/30/15 by vol), and the solvent was removed by rotary evaporation at 35 °C. The residue was dissolved in 1,2-dichloroethane and transferred into a glass-stoppered test tube. The solvent was removed under a stream of nitrogen, and the residue was then redissolved in 0.3 mL of acetonitrile/2-propanol (1/1 v/v). The sample was applied to a Sep-Pak C_{18} cartridge, prewashed before sample application with 3 mL of acetonitrile followed by 5 mL of acetonitrile/2-propanol/water (55/25/20 by vol). Cholesterol oxides were then eluted by washing the column with 16 mL of acetonitrile/2-propanol/water (55/25/20 by vol). Subsequently, the cholesterol oxide fraction was dried in a rotary evaporator, and the residue was dissolved in 1,2-dichloroethane, transferred into a glass-stoppered test tube, and kept at 4 °C. Prior to HPLC, the solvent was removed under a stream of nitrogen and the sample dissolved in 60 μL of heptane/2-propanol (95/5 v/v). The normal phase separation was performed on a Hibar, Lichrosorb CN (5 μm) column, 250 mm × 4 mm (E. Merck, Darmstadt, FRG) using heptane/2-propanol (95/5 v/v) as a mobile phase and a flow rate of 0.5 mL/min. The cholesterol oxide fraction was collected commencing at the start of the 5α,6α-epoxycholesterol peak and concluding after elution of the cholestanetriol peak. The solvent was removed by rotary evaporation, and the residue was transferred into a glass-stoppered test tube and kept at −20 °C until derivatization. A scheme for the analytical procedure is presented in Figure 1.

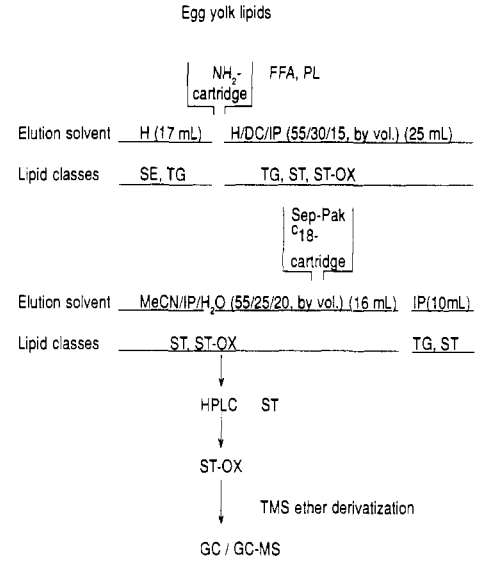

Figure 1. Enrichment and final determination of the autoxidation products from free cholesterol. Abbreviations: H, hexane; DC, 1,2-dichloroethane; IP, 2-propanol; MeCN, acetonitrile; SE, sterol esters; TG, triacylglycerols; ST, sterols; ST-OX, sterol oxides; PL, phospholipids; FFA, free fatty acids.

Enzymatic Hydrolysis of Triacylglycerols and Esterified Cholesterol. (*A*) *Model System. Substrate Preparation.* A substrate consisting of tripalmitolein (1.0 mg), cholesteryl oleate (0.05 mg), and cholesterol (0.1 mg) was transferred into a glass test tube, and the solvent was removed under a stream of nitrogen. Five milligrams of detergent (bovine serum albumin), followed by 1 mL of phosphate buffer (5 mM; pH 9.1) was added. The sample was sonicated for 1 min by using a Rapidis 150 sonicator (Ultrasonics Ltd., Sheplid, Yorkshire, U.K.) at a power level setting of 5. This is essentially a scaled-down version of the procedure used for analysis of the cholesterol oxides in authentic samples, as described below.

Triacylglycerol Hydrolysis. Eighty microliters of triacylglycerol lipase (1.8 units of enzyme/μL) was added to the emulsified substrate. The sample was then incubated at room temperature under vigorous shaking for 6 h. The enzyme reaction was terminated by adding 1 mL of deionized, distilled water and 4 mL of absolute ethanol (99.9%). The mixture was then shaken vigorously for about 30 s, and 2 mL of 1,2-dichloroethane containing 0.2 mg of triheptadecanoin (TG 17:0) was added as an internal standard. The suspension was then shaken for another 30 s and allowed to settle, and the lower layer was transferred into a glass-stoppered test tube. The ethanolic phase was reextracted twice with 2 mL of 1,2-dichloroethane, the lower layers were pooled, and the solvent was removed under a stream of nitrogen. Subsequently, the residue was redissolved in 200 μL of chloroform, applied on a 1-cm band on a silica gel 60 thin-layer chromatography (TLC) plate, and then developed. The reference band was visualized by exposing the reference portion of the TLC plate to iodine vapor, and the residual unhydrolyzed triacylglycerol zone was rapidly scraped off and transferred into a 10-mL glass-stoppered test tube for immediate derivatization to fatty acid methyl esters, according to the method of Appelqvist (1968). In short, 3 mL of 10 mM NaOH in dry methanol was added, and the sample was heated for 30 min at 60 °C. Water (1.5 mL), followed by 2 mL of hexane, was added, and the mixture was shaken. The upper layer, mainly hexane, was transferred into a new glass-stoppered test tube, and the solvent was removed under a stream of nitrogen. Finally, the residue was dissolved in 50 μL of hexane, and 1–2 μL were injected into the gas chromatograph.

Cholesterol Ester Hydrolysis. The emulsified substrate (see Substrate Preparation) was incubated with 80 μL of triacylglycerol lipase (1.8 units/μL) for 6 h under vigorous shaking. The reaction was terminated by added 1 mL of deionized, distilled water and 4 mL of absolute ethanol, and the mixture was shaken for 30 s. 1,2-Dichloroethane (2 mL) was added, the suspension was again shaken, and the lower layer was transferred into

a glass-stoppered test tube. Subsequently, the ethanolic layer was reextracted twice with 1,2-dichloroethane, the lower layers were pooled, and the solvent was removed under a stream of nitrogen. The residue was redissolved in 1 mL of hexane/1,2-dichloroethane (1/1 v/v) and applied on a NH_2 cartridge to remove the released free fatty acid. The remaining lipids were eluted with 30 mL of hexane/1,2-dichloroethane/2-propanol (55/30/15 by vol), and the solvent was removed by rotary evaporation. The residue was dissolved in 1,2-dichloroethane and transferred into a glass test tube, and the solvent was removed under a stream of nitrogen. Bovine serum albumin (5 mg) followed by 2 mL of phosphate buffer was added, and the sample was sonicated. Cholesterol ester hydrolase (200 μL containing 11 units of enzyme/μL) was added, and the emulsified sample was incubated at room temperature for 3 h under vigorous shaking. The enzyme reaction was stopped by the addition of 1 mL of water and 4 mL of absolute ethanol, the mixture was shaken, and the lipids were extracted with 1,2-dichloroethane as described above. The solvent was dried under a stream of nitrogen, and the residue was dissolved in 200 μL of chloroform. The sample was applied to a silica gel 60 TLC plate and developed.

(*B*) *Authenic Sample.* Approximately 0.8 μg of 3β-hydroxycholest-5-en-22-one (internal standard) was added to 25 mg of egg yolk lipids in 1,2-dichloroethane, and the solvent was removed under a stream of nitrogen. Twenty milligrams of bovine serum albumin followed by 25 mL of phosphate buffer was added, and the sample was sonicated (see Substrate Preparation). Triacylglycerol lipase solution (300 μL containing 72 units of enzyme/μL) was added, and the emulsified sample was incubated under vigorous shaking for 6 h. The sample was then transferred into a separation funnel, 50 mL of ethanol was added, and the mixture was shaken for 30 s. 1,2-Dichloroethane (15 mL) was then added, the suspension was again shaken, and the lower layer was transferred into an evaporation flask. The ethanolic phase was extracted twice with 1,2-dichloroethane, the lowewr layers were pooled, and the solvent was removed in a rotary evaporator. The residue was dissolved in 1,2-dichloroethane, transferred into a glass-stoppered test tube, dried under a stream of nitrogen, and then redissolved in 1 mL of hexane/1,2-dichloroethane (1/1 v/v). The sample was applied to a NH_2 cartridge to remove the phospholipids and released fatty acids. The remaining lipids were eluted by washing the column with 30 mL of hexane/1,2-dichloroethane/2-propanol (55/30/15 v/v/v) and dried in a rotary evaporator. The residue was dissolved in 1,2-dichloroethane and then transferred into a glass-stoppered test tube, where the solvent was removed under a stream of nitrogen. Ten milligrams of bovine serum albumin followed by 10 mL of phosphate buffer was added, and the sample was again sonicated. Cholesterol ester hydrolase (250 μL containing 11 units of enzyme/μL) was added, and the emulsified sample was shaken vigorously for 3 h. The reaction was terminated by adding 15 mL of deionized, distilled water followed by 50 mL of absolute ethanol. The lipids were extracted with 1,2-dichloroethane, essentially as described above, and the solvent was removed in a rotary evaporator. The residue was dissolved in 1,2-dichloroethane and transferred into a glass-stoppered test tube followed by removal of the solvent under a stream of nitrogen. The residue was redissolved in 1 mL of hexane/1,2-dichloroethane (1/1 v/v) and then applied to a new NH_2 cartridge. The remaining procedure was essentially the same as that described for the enrichment of the autoxidation products from free cholesterol (Figure 1).

Thin-Layer Chromatography (TLC). TLC was performed by using silica gel 60 plates, 20 × 20 cm, 0.25-mm thickness (E. Merck). The samples, and a reference mixture, were applied by using the automatic applicator Linomat III (CAMAG, Muttenz, FRG). The TLC plate was developed in toluene/methanol (1/1 v/v) to 5 cm from the starting line and dried in a ventilated cabinet at room temperature. The plate was then rerun in toluene to 16 cm and dried at room temperature before visualization of the bands. For tri-, di-, and monoacylglycerols and free fatty acid analysis, the bands were visualized by staining in a tank with iodine vapors. For the detection of cholesteryl ester, cholesterol, cholesterol oxides, and phospholipids the TLC plate was sprayed with 50% H_2SO_4 and heated at 110 °C for 5 min for color development.

High-Performance Liquid Chromatography (HPLC). HPLC was carried out by using a Spectra Physics HPLC system, consisting of a Model SP 8700 solvent delivery system, a Model SP 8700 pump, a Model R 401 Waters differential refractometer, and a Rheodyne injector fitted with a 50-μL sample loop.

Derivatization of the Sterol Oxides to Trimethylsilyl Ethers (TMS Ethers). The cholesterol oxide fraction was dried under a stream of nitrogen, Tri-Sil (100 μL) was added, and the residue was dissolved in the TMS reagent by shaking. The sample was subsequently heated at 60 °C for 30 min. The solvent was removed under a stream of nitrogen and the sample redissolved in 100 μL of hexane. One microliter of the sample was then injected into the gas chromatograph.

Gas Chromatography (GC) and Gas Chromatography–Mass Spectrometry (GC–MS). Sterols were analyzed by using a Varian 3700 gas chromatograph equipped with a flame ionization detector and a falling needle injector. The cholesterol oxides were separated as TMS ethers on a chemically bonded methyl silicone column, 25 m × 0.2 mm, film thickness 0.2 μm (Quadrex Corp., New Haven, CT). Operating parameters were as follows: column temperature 250 °C; detector temperature 310 °C, inlet pressure 14 psi; and helium as carrier gas.

The fatty acid methyl esters were analyzed on a Pye Unicam GCD gas chromatograph equipped with a flame ionization detector and a glass column (2.1 m × 2 mm) packed with 10% butanediol succinate on Chromosorb AWHP (100–120 mesh). Operation conditions were as follows: column temperature 200 °C; injector temperature 250 °C; and detector temperature 250 °C. Nitrogen was used as carrier gas. The peaks were recorded by using a 3990A HP computing integrator.

Mass spectrometry identification was performed by using a Finnigan TSQ 70 GC–MS equipped with a falling needle injector system and the previously mentioned capillary methyl silicone column. Operating parameters were as follows: column temperature 230 °C; interface temperature 250 °C. The spectra were obtained by using electron impact ionization at 70 eV and a scan rate of 1 s/scan.

Identification of the cholesterol oxides was made by comparison of the relative retention time values and total ion mass spectra of the components in the sample of those of eight synthetic cholesterol oxides. The cholesterol oxides were quantified by area measurement of the sample peaks relative to that of the internal standard, 22-ketocholesterol.

RESULTS AND DISCUSSION

Chromatographic Studies. Triacylglycerols, phospholipids, and cholesterol are the major components in egg yolk lipids, 87%, 10%, and 2%, respectively. About 90% of the total cholesterol is in the free form. To remove the triacylglycerols and the phospholipids in a single step, a disposable NH_2 cartridge was tested by using various solvent systems. When the NH_2 cartridge was charged with 100–150 mg of egg yolk lipids containing 5α,6α-epoxycholestanol and/or cholestanetriol, thin-layer chromatography showed that the triacylglycerols were almost entirely removed by washing the NH_2 cartridge with 17 mL of hexane, while the cholesterol and the cholesterol oxides were eluted by washing the column with 25 mL of hexane/1,2-dichloroethane/2-propanol (55/30/15 by vol). The phospholipids were, however, retained on the column by using this enrichment step. To ascertain the capacity for retaining phospholipids and free fatty acids, 20 mg of egg yolk phospholipids and/or 30 mg of linoleic acid (18:2) were applied to a NH_2 cartridge. TLC analysis of the fractions from the NH_2 cartridge demonstrated that free fatty acids and phospholipids were retained in their entirety on the column at this load level.

Attaining greater resolution during gas chromatography analysis of cholesterol oxides requires removal of the cholesterol. The efficiency of various solvent systems in

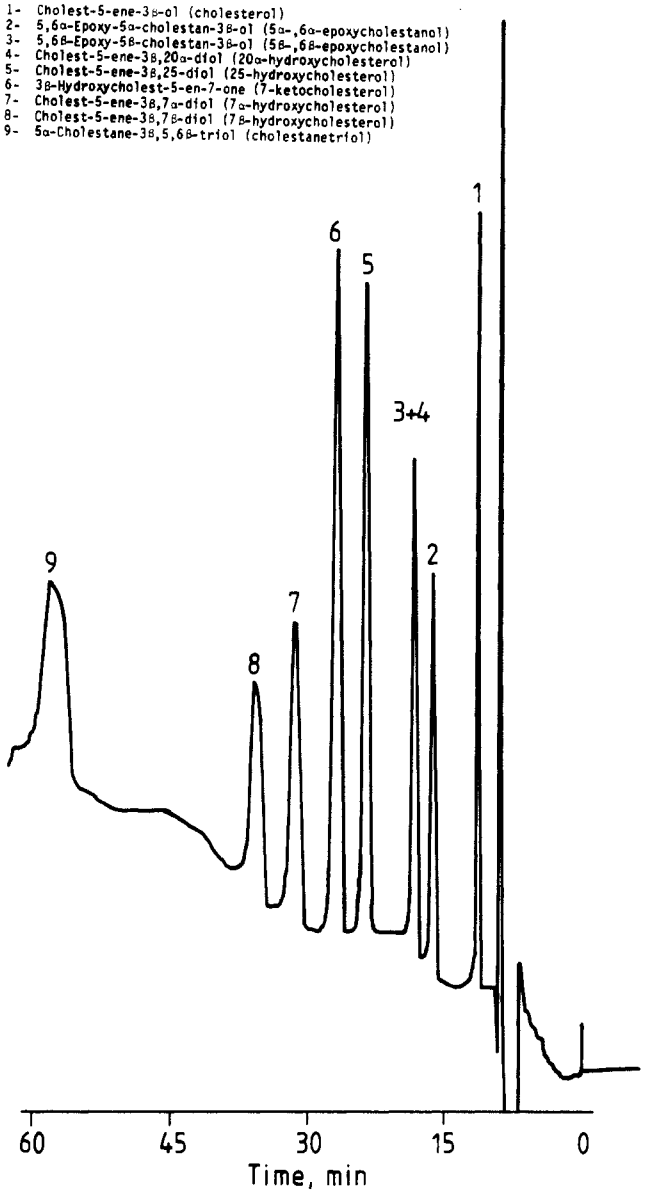

Figure 2. HPLC separation of synthetic cholesterol oxides on a Lichrosorb CN column (250 mm × 4 mm) using hexane/2-propanol (95/5 v/v) at the flow rate 0.4 mL/min.

removing the major part of a fixed amount of cholesterol applied to a Sep-Pak C_{18} cartridge was explored. A solvent mixture consisting of acetonitrile/2-propanol/water in the proportion 55/25/20 by volume was found to give an optimal retention of the added compound as revealed by TLC analysis. Analysis of a cholesterol oxide fraction from authentic sample after NH_2 enrichment steps indicated that a major part of the cholesterol and the remaining triacylglycerols were retained on the column when it was washed with 16 mL of acetonitrile/2-propanol/water (55/25/20 by vol). Before such preparative steps were applied to actual samples, it was necessary to estimate the recoveries for ^3H-labeled cholestanetriol during the NH_2 chromatography and for the ^3H-labeled cholesterol and the ^{14}C-labeled epoxycholestanol in the reversed-phase chromatography (Sep-Pak C_{18}). The recovery for [^3H]-cholestanetriol was 83 ± 7% (n = 4), that for [^{14}C]-epoxycholestanol 98 ± 2% (n = 4), and that for [^3H]-cholesterol 40 ± 5% (n = 4). Hence, it is obvious that the sequential use of an NH_2 cartridge and a Sep-Pak C_{18} cartridge is a convenient and highly effective way to enrich the cholesterol oxides in egg yolk lipids.

HPLC on normal-phase or reversed-phase column materials has been used in several investigations for the

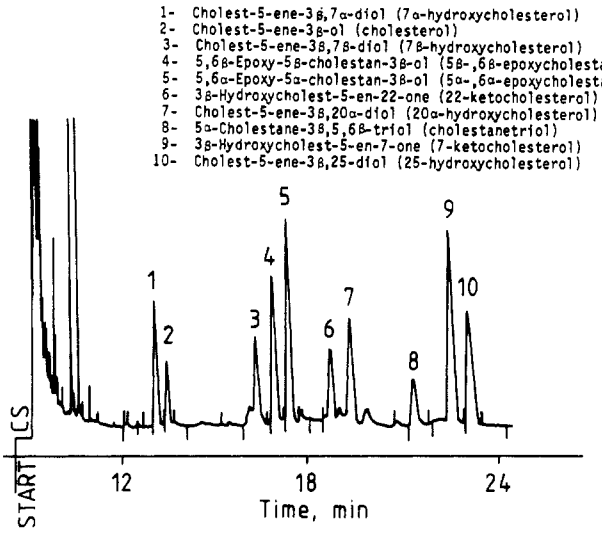

Figure 3. Separation of TMS ether derivatives of synthetic cholesterol oxides on a capillary methyl silicone column (25 m × 0.25 mm; film thickness 0.25 μm).

quantitative determination of certain cholesterol oxides, viz., the isomeric 5,6-epoxycholestanols, C-7 oxidized cholesterols, and 25-hydroxycholesterol (Tsai and Hudson, 1984; Herian and Lee, 1985; Lee et al., 1985; Park and Addis, 1985; Kou et al., 1985; Sugino et al., 1986). Preparative HPLC on silicic acid column was used by Missler et al. (1985) to remove the bulk of cholesterol from the cholesterol oxide fraction obtained from egg yolk mix powder. Missler et al. (1985) reported low analyzing capacity due to the "memory effects" encountered during HPLC analysis, which required the column to be cleaned after each sampling.

In the present study, a cyanopropyl column was used for the removal of the remaining cholesterol from the autoxidation products. Figure 2 shows the order of elution of eight interesting cholesterol oxides analyzed in this investigation. These were eluted in the order reported by Shen and Sheppard (1983) for normal-phase operation mode using a μPorasil column, with the exception of the isomeric 5,6-epoxycholestanols which were eluted prior to 25-hydroxycholesterol. The cyanopropyl column was selected because (a) it is superior to silica in separating the cholesterol from the side-chain hydroxylated cholesterols, viz., 20α-hydroxycholesterol and 25-hydroxycholesterol, and (b) relatively short retention times were needed for a wide range of cholesterol oxides including cholestanetriol. It should be mentioned that interfering peaks were observed in the HPLC chromatogram of the authentic samples in spite of the NH_2 sorbent and Sep-Pak C_{18} enrichment steps. None of these however, overlapped with the elution times of cholesterol oxides or influenced the final GC separation.

The 22-ketocholesterol was selected as an internal standard, by virture of (a) its intermediate relative retention time value as TMS ether on a GC capillary methyl silicone column (Figure 3) and (b) its elution order, immediately after the 5α,6α-epoxycholestanol peak on the cyanopropyl column during the HPLC analysis (Figure 2). Moreover, GC analysis showed that 87 ± 14% (n = 4) of the added 22-ketocholesterol was recovered after the NH_2 and C_{18} enrichment steps using cholestane as an internal standard. The GC response factor at the load interval 100–800 ng was 0.85 (n = 2), with that of cholestane being 1.00. However, no correction for possible differences in response factor was made for the sample cholesterol oxides analyzed in this system. The response factors were assumed to be

Table I. Autoxidation Products from Free Cholesterol in Fresh Egg Yolk and Dehydrated Egg Yolk

product	cholesterol oxides,[a] ppm in the lipids[b]				
	5α,6α-epoxy-cholestanol	5β,6β-epoxy-cholestanol	7-keto-cholesterol	7α-hydroxy-cholesterol	7β-hydroxy-cholesterol
fresh egg yolk (exp 1)[c]	ND[d]	ND	2.2	2.7	ND
fresh egg yolk (exp 2)[c]	ND	ND	1.9	2.6	ND
dehydrated egg yolk[e]	5.2	11.7	3.0	5.5	NQ[f]

[a] The 20α-hydroxycholesterol, 25-hydroxycholesterol, and cholestanetriol were not detected. [b] The values are mean of duplicate analyses. [c] Experiment 1, without nitrogen protection; experiment 2, enrichment on aminopropyl cartridge and Sep-Pak C_{18} enrichment were performed under nitrogen atmosphere. [d] Not detected; detection limit 0.5 ppm in the lipids. [e] The sample was stored at 18–22 °C for 9 months and then for 14 months at −20 °C. [f] Not quantifiable because of an interfering compound.

Table II. Autoxidation Products from Free and Esterified Cholesterol in Fresh Egg Yolk

expt[a]	cholesterol oxides,[b] ppm in the lipids				
	5α,6α-epoxy-cholestanol	5β,6β-epoxy-cholestanol	7-keto-cholesterol	7α-hydroxy-cholesterol	7β-hydroxy-cholesterol
1	19.5	83.8	24.9	13.0	NQ[c]
2	7.4	17.4	4.6	8.0	NQ
3	ND[d]	ND	ND	TR[e]	ND
4	TR	TR	ND	ND	ND

[a] Experiment 1, without nitrogen protection; experiment 2, flushed with nitrogen immediately before the enzymatic hydrolysis steps; experiments 3 and 4, enzymatic hydrolysis steps under nitrogen atmosphere. The figures in experiments 1–3 were based on single analyses, while the figures in experiment 4 were based on duplicate analyses. [b] The 20α-hydroxycholesterol, 25-hydroxycholesterols, and cholestanetriol were not detected. [c] Not quantifiable because of interfering compound. [d] Not detected; detection limit 2 ppm in the lipids. [e] Traces (<2 ppm in the lipids).

close to 1.00, on the basis of the relative response factor estimation performed by Park and Addis (1985) for TMS ethers of cholesterol oxides.

Studies on the Enzymatic Hydrolysis of Triacylglycerols Followed by Enzymatic Release of Esterified Cholesterol To Facilitate Estimation of Sum of Free and Esterified Cholesterol Oxides. In the incubation of the substrate (1.0 mg of triacylglycerol TG 16:1, 0.05 mg of cholesteryl oleate, and 0.1 mg of cholesterol) with triacylglycerol lipase for 1, 2, 3, and 4 h, it was found that the bulk of the triacylglycerol was hydrolyzed to free fatty acids after 4 h, as measured by the intensity of the bands after TLC analysis. However, a longer incubation time was required to achieve a maximal hydrolysis level. The experiments were repeated in duplicate at different incubation times (3, 4, 5, and 6 h), and the unhydrolyzed triacylglycerol bands were derivatized to fatty acid methyl esters and were then analyzed by GC. The amounts of residual triacylglycerols at different incubation times were approximately 17%, 7%, 7%, and 2%, respectively, of that added. The differences between the 4-, 5-, and 6-h incubation times is probably due to variation in the sonication of the substrates rather than differences in incubation period. The amounts of mono- and diacylglycerols were not estimated since TLC analysis demonstrated that the levels of these components were very low compared to the level of the remaining unhydrolyzed triacylglycerols.

To study the hydrolysis of cholesteryl esters, a substrate consisting of only cholesterol (0.1 mg) and cholesteryl oleate (0.05 mg) was incubated with cholesterol esterase for 1 and 3 h. TLC analysis revealed the presence of very low levels of cholesteryl oleate after a 3-h incubation, whereas after 1 h, the major part of the cholesterol oleate was detected unchanged. When the experiment was repeated in the presence of 1.0 mg of linoleic acid, TLC analysis revealed a slight increase in the intensity of the cholesteryl oleate zones compared to the control. It appears that, in the presence of a relatively large amount of free fatty acids, the reaction favors esterification rather than hydrolysis of cholesteryl oleate. Therefore, the use of a NH_2 chromatography step before the cholesteryl ester hydrolysis was essential to remove the fatty acids resulting from the hydrolysis of the triacylglycerols.

The efficiency of cholesteryl ester hydrolysis in the authentic sample was estimated by added ^{14}C-labeled cholesteryl oleate. After the triacylglycerol and cholesteryl ester hydrolysis steps, the remaining lipid components were separated on a silica gel 60 TLC plate, and the cholesteryl oleate and cholesterol zones were removed and assayed for ^{14}C activity. Two percent of the total [^{14}C]-cholesteryl oleate (n = 4) was recovered, while the corresponding recoveries for the released [^{14}C]cholesterol were as high as 90–95%. It is apparent that the enzymatic hydrolysis of the cholesteryl esters is a highly effective step in total cholesterol determination. In the present investigation, no attempt was made to determine the hydrolysis rate of different esterified cholesterol oxides. Cholesterol esterase specifically attacks the functional group at the carbon atom at the 3β-position. On this basis, the hydrolysis ratio of esterified cholesterol to esterified cholesterol oxide was assumed to be close to 1. It may, therefore, be suggested as an alternative to cold saponification for total cholesterol oxide analysis.

Levels of Cholesterol Oxides in Egg Yolk Products. Analysis of a fresh egg yolk sample for free cholesterol oxides indicated the presence of quantifiable levels of the 7α-hydroxycholesterol and 7-ketocholesterol at the detection limit 0.5 ppm in the lipids (experiment 1, Table I). To determine the level of artifact (if generated) during the NH_2 and Sep-Pak C_{18} enrichment steps, the analysis was performed under nitrogen protection. The analytical data (experiment 2, Table I) revealed no differences compared to the control (experiment 1, Table I). The absence of the isomeric 5,6-epoxycholestanols, 7β-hydroxycholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, and cholestanetriol was established by GC–MS analysis using the multiple ion detection method. The analyses were performed in duplicate (from extraction to derivatization of the cholesterol oxide fraction) in about 6 h.

Nourooz-Zadeh and Appelqvist (1987) reported that none of the eight cholesterol oxides of interest was observed in fresh egg yolk at the detection limit 0.2 ppm in the lipids, while only a trace of 7-ketocholesterol was detected in freeze-dried egg yolk powder. Tsai and Hudson (1984) reported the absence of the isomeric 5,6-epoxycholestanols in freeze-dried egg yolk powder at the detection limit

0.5 ppm in the lipids. The 7α-hydroxycholesterol and 7-ketocholesterol are known intermediates during in vivo degradation of cholesterol in mammals (Smith, 1981). Therefore, it is probable that the finding of these cholesterol oxides in the present sample of fresh egg yolk lipids is due to deposition of metabolites of cholesterol in the egg rather than their accumulation during the analytical procedure. Hence, further studies should be conducted to ascertain the reasons for the discrepancy between results on fresh eggs in the present study and those reported by Nourooz-Zadeh and Appelqvist (1987).

Further, a spray-dried egg yolk powder sample, stored for 9 months at 18–22 °C and then for about 14 months at −20 °C, was analyzed. The sample contained substantial levels of certain cholesterol oxides, viz., the isomeric 5,6-epoxycholestanols and the C-7 oxidized cholesterol, but none of the other cholesterol oxides of interest (Table I).

Before the enzymatic method was applied, it was essential to evaluate the levels of artifacts without use of a nitrogen atmosphere during the enzymatic hydrolysis steps. Analysis of fresh egg yolk revealed the accumulation in the lipids of 141.3 ppm of total cholesterol oxides (experiment 1, Table II). The 5β,6β-epoxycholestanol was the major component followed in decreasing order by 5α,6α-epoxycholestanol, 7-ketocholesterol, and the epimeric 7-hydroxycholesterols. Therefore, the major part of the method development was directed to minimizing the generation of artifacts during the analytical procedure before its utilization for routine purposes.

Flushing the substrate with nitrogen immediately before the triacylglycerol and cholesteryl ester hydrolysis steps (experiment 2, Table II) caused an obvious decrease in the level of artifacts from 141.3 to 37.4 ppm in the lipids. Performing the enzymatic hydrolysis steps under continuous nitrogen flushing at the flow rate 30 mL/min revealed a complete inhibition of artifact formation during the analytical procedure (experiment 3, Table II). To ascertain the absence of artifacts, experiment 3 was repeated in duplicate. The analysis indicated the presence of trace levels of the isomeric 5,6-epoxycholestanols at the detection limit 2.0 ppm in the lipids. The analyses were performed in dim light without interpretation from lipid extraction to TMS ether derivatization (about 19 h).

Fischer et al. (1985) reported the presence of about 10 ppm of the isomeric 5,6-epoxycholestanols, 9 ppm of 7α-hydroxycholesterol, and 15 ppm of 7β-hydroxycholesterol in lipids from two freeze-dried whole egg samples, using hot saponification as a preconcentration step. Apparently, the finding of relatively high levels of the predominant cholesterol oxides in the freeze-dried whole egg samples is due to generation of artifacts during the analytical procedure used by Fischer et al. (1985) rather than a contribution from the small amount of cholesteryl esters in the egg yolk lipids.

An attempt was made to apply the cold saponification according to the method of Park and Addis (1986) as an alternative to the laborious enzymatic method. Analysis of the unsaponifiable material obtained from a fresh egg yolk extract by cold saponification indicated the presence of slightly higher levels of the C-7 oxidized cholesterols compared to the control, a fresh egg yolk analyzed only for the content of the oxides from free cholesterol. Futher studies should be conducted to investigate the source of the increased level of the oxides after cold saponification to determine if it derives from the esterified cholesterol or is an artifact from the analytical procedure.

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Registry No. 5α,6α-Epoxycholestanol, 1250-95-9; 5β,6β-epoxycholestanol, 4025-59-6; 7-ketocholestanol, 7591-17-5; 7α-hydroxycholesterol, 566-26-7; 7β-hydroxycholesterol, 566-27-8; 20α-hydroxycholesterol, 516-72-3; 2S-hydroxycholesterol, 2140-46-7; cholestanetriol, 72879-16-4; triacylglycerol lipase, 9001-62-1; cholesterol ester hydrolase, 9026-00-0; 22-ketocholesterol, 19243-30-2.